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Optimization of extraction process for phenolic acids from black cohosh (*Cimicifuga racemosa*) by pressurized liquid extraction†

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Abstract: An investigation to optimize the extraction of phenolic acids from black cohosh using a pressurized liquid extractor system was studied with the aim of developing a generalized approach for sample preparation of phenolic compounds from plant matrices. Operating parameters such as solvent composition, solid-to-solvent ratio, temperature, particle size distribution, and number of extraction cycles were identified as main variables that influence extraction efficiency. A mixture of methanol and water (60:40 v/v) was found to be the best solvent for total phenolics (TP) and individual phenolic acids. The four phenolic acids extracted from black cohosh were identified by HPLC and LC-MS as caffeic acid, ferulic acid, sinapic acid and isoferulic acid. Over 96% of the measured phenolics were extracted in first two cycles. The extraction efficiency for black cohosh with MeOH:H_2O (60:40 v/v) was found to be maximum at a solid-to-solvent ratio of 80 mg ml^{-1}. TP content of the extract was found to increase with temperature up to 90 °C. Particle size was found to have a large impact on extraction efficiency of TP. Samples with particle size between 0.25 mm and 0.425 mm provided optimum extraction of phenolics from black cohosh.
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Keywords: total phenolics; phenolic acids; black cohosh; *Cimicifuga racemosa*; pressurized liquid extraction; sample preparation; solvent composition; solid-to-solvent ratio; particle size distribution; Folin–Ciocalteu; HPLC; LC-MS

INTRODUCTION

Phenolics are a large class of secondary plant metabolites ubiquitous in the plant kingdom. The term 'phenolics' encompasses approximately 8000 naturally occurring compounds all of which possess one common structural feature: a phenol (an aromatic ring bearing at least one hydroxyl substituent). Polyphenols, possessing two or more phenol subunits, include the flavonoids and tannins. The phenolic compounds are of considerable interest in a variety of fields including plant biochemistry, physiology,[1–4] organoleptic chemistry[5] and food technology.[6] More recent interest in these phenolic plant components stems from their purported health benefits. They have been associated with the inhibition of oxidative damage diseases such as coronary heart disease, stroke, and cancers.[7] Robust analytical methodologies are needed for the accurate determination of phenolics concentrations in plant material.

Sample preparation (SP) is a term that encompasses a variety of steps ranging from sample grinding, exhaustive solvent extraction and preconcentration procedures to simple liquid–liquid extraction or filtration. Certain phenolics are not 'free' in the plant matrix, instead they are bound to larger molecules and even cell walls, so that liberating these phenolics, via hydrolysis for example, can be a part of the SP.[8] In many literature reports, SP is often seen as 'a means to an end' where emphasis is placed on the instrumentation (chromatography and spectroscopic).

Extraction is, in large part, an equilibrium controlled process where the goal is to provide isolation and enrichment of analytes from a permeable plant material.[9] One factor in a successful SP sequence for phenolics would, ideally, involve an exhaustive and reproducible extraction of analytes from the plant matrices. Because of wide variations in structures and polarities of the phenolic compounds, extraction of phenolic compounds from plant matrices is complex and challenging. Unfortunately, optimization of many of the critical extraction parameters (e.g. solvent, time, solid-to-solvent ratio, number of extractions, temperature and particle size of sample material) involved with SP, is not the focus of many investigations.[10,11] For accurate, reproducible and quantitative measurements of phenolic compounds, SP is of critical importance since reports estimate that approximately 30% of the error in analytical measurements comes from the sample preparation

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steps.[12,13] Yet, despite the increased research interest in phenolic compounds due to their potential health benefits,[14,15] there is no definitive method for SP of phenolic compounds at this time.

With the aim of finding optimal conditions for the extraction of phenolic acids, we investigated the effect of solvent composition, the solid-to-solvent (S/L) ratio, number of extraction steps, temperature, pressure, and particle size distribution on the extraction efficiency of phenolics using an accelerated solvent extractor (ASE). A similar approach can also be extended to optimization of other classes of phenolic compounds (flavanones, tannins, anthocyanidins, flavonols, isoflavones and other micronutrients) present in various food matrices. During the extraction process, analytes are distributed between the extracting solvent and solid plant material until an equilibrium between the concentration of the analytes in both phases is reached. ASE allows the use of elevated temperature in combination with pressure to enhance this molecular interaction and increase removal of analytes from solid and semisolid matrices. Since ASE is a commercial designation that does not necessarily bear a relationship to the basis of the technique, the term PLE (pressurized liquid extraction) will be used in the remainder of this paper.[16]

Black cohosh (*Cimicifuga racemosa*) was used as a model plant material in this study as extracts of black cohosh are used for the treatment of menopausal disorders and black cohosh is one of the dietary supplements that is currently under evaluation by the Office of Dietary Supplement (ODS), National Institute of Health (NIH). Black cohosh, although well-known for its triterpene glycoside content,[17,18] is a rich source of phenolic acids and polyphenols.[19] Although neither the mode of action nor the active constituents are known, the hormonal activity of black cohosh has been attributed to both the lipophilic and phenolic fractions (hydroxycinnamic acid derivatives) of the extract.[18]

The objectives of this paper is to study the influence of key parameters such as extraction solvent polarity, number of extraction cycles, solid-to-solvent ratio, particle size, temperature and pressure on extraction of TP from black cohosh in order to develop a generalized sample preparation methodology for extraction of phenolic acids.

MATERIALS AND METHODS

Plant material

Fresh freeze-dried powder of black cohosh from root and rhizome (Wild Harvest Grade C Powder Run #947) was obtained from Dr David Lytle of the Eclectic Institute, Sandy, Oregon, USA, in June 2003. Soon after receipt, material was stored in a freezer at −62 °C.

Chemicals

HPLC-grade methanol (MeOH), acetonitrile (ACN), diatomaceous earth (Celite 545) and Ottawa sand were purchased from Fisher Chemicals (Fair Lawn, NJ, USA). HPLC-grade dimethyl sulfoxide (DMSO) and acetone were purchased from Sigma-Aldrich (St Louis, MO, USA) and Burdick & Jackson (Muskegon, MI, USA), respectively. Denatured anhydrous ethanol (EtOH) was obtained from Mallinckrodt (Paris, KY, USA), and tetrahydrofuran (THF) was purchased from Aldrich Chemicals (Milwaukee, WI, USA). Folin–Ciocalteu (FC) reagent, gallic acid, and sodium carbonate for the assay of total phenolics (TP) were obtained from Sigma Chemicals (St Louis, MO, USA). Deionized water (18 Ω) was prepared using a Millipore Milli-Q purification system (Millipore Corp., New Bedford, MA, USA).

Methodology of extraction

Black cohosh powder was extracted with different solvents using a PLE (Model ASE 200, Dionex Corporation, Sunnyvale, CA, USA). Aliquots of freeze-dried black cohosh powder were mixed with Celite 545 (a drying and dispersing agent) in a 4:1 proportion and placed in a 33-ml stainless-steel extraction cell. The void volume in the cell was filled with Ottawa sand.

Extraction of black cohosh was carried out using two extraction cycles with the experimental solvent at 40 °C and 1000 psi for 5 min. The cell was then rinsed with fresh solvent (half of the cell volume) and purged with a flow of nitrogen (purge time = 90 s) drawing the extract into a 60-ml amber glass vial with Teflon-coated rubber caps (I-CHEM, New Castle, DE, USA). Unless otherwise mentioned, all extraction experiments were carried out under these default conditions. Samples were kept in the dark in a freezer before analysis. Each extract was filtered through a 0.45-μm polyvinylidene filter (PVDF) (National Scientific Company, Duluth, GA, USA) prior to analysis.

To study the influence of different sample preparation parameters, extractions and analyses were carried out in triplicate with each experiment. The amount of black cohosh used in each extraction was 0.660 g.

Solvent composition

Several neat solvents [MeOH, EtOH, deionized H_2O, acetonitrile (ACN), tetrahydrofuran (THF), acetone, and DMSO], as well as a systematic variation of ratios of MeOH to H_2O ranging from 1:1 to 0:1 (v/v) MeOH, in increments of 0.1, were used for extraction of phenolics from black cohosh. A comparison of the aqueous influence on extraction of phenolics was carried out using 1:1 (v/v) mixtures of solvents (MeOH, EtOH, acetone and DMSO) with H_2O.

Number of extraction steps

In these experiments, the number of cycles was varied between one and three. Black cohosh was extracted with MeOH:H_2O (60:40, v/v) solvent mixture. Extracts from each cycle were collected in separate extraction vials.

Solid-to-solvent ratios

In these experiments, the amount of black cohosh was varied between 0.165 g and 6.6 g to obtain seven different S/L ratios ranging between 5 and 200 mg mL^{-1}. Extractions were carried out with MeOH:H_2O (60:40, v/v) solvent mixture.

Temperature effect

Temperature influence on extraction was examined by conducting experiments at different temperatures (40–100 °C) at 1000 psi. Extractions were carried out with MeOH:H_2O (60:40, v/v) solvent mixture.

Particle size distribution

Influence of particle size on extraction was investigated by sieving the freeze-dried black cohosh powder using standard sieves (numbers 10, 20, 40, and 60 corresponding to opening sizes of 2, 0.85, 0.425, and 0.250 mm, respectively). Freeze-dried black cohosh powder was sieved vigorously while enclosed with sieve cover and pan.[20] Five different fractions were collected at the end of the sieving process. These fractions were used to study the influence of particle size on extractability with MeOH:H_2O (60:40, v/v) solvent mixture.

Pressure dependence

Extractions of black cohosh were conducted at three different pressures (500, 1000 and 1500 psi) using MeOH:H_2O (60:40, v/v) solvent mixture at 40 °C. Other operating parameters were kept unchanged during the experiments.

Estimation of total phenolics (TP)

The TP content was determined using the Folin–Ciocalteu (FC) assay with gallic acid as a standard in a Perkin-Elmer (Boston, MA, USA) Lambda 25 spectrophotometer.[21] A calibration curve was created using standard gallic acid solutions each time an analysis was run. The level of TP in the extract was calculated from the standard calibration curve. Results were expressed in mg gallic acid equivalent per gram of dry black cohosh (mgGAE g^{-1}).

Separation of phenolic acids by HPLC

Black cohosh extracts were analyzed by using a HPLC system (Beckman Coulter, Fullerton, CA, USA; System Gold) coupled with a programmable detector (System Gold, series 166) and an autosampler (System Gold, series 508) operated by a 32 Karat software package. A reversed phase C_{18} Luna column (Phenomenex, Torrance, CA, USA; 150 × 4.6 mm; particle size 5 μm), preceded by a guard column (Phenomenex, 4 × 3.0 mm) of the same stationary phase was used for HPLC and LC-MS analysis. The column and the guard column were thermostatically controlled at 25 °C and the flow rate was set to 0.7 mL min^{-1}. The mobile phase consisted of two solvents; 1 g L^{-1} formic acid (A) and methanol (B). The solvent gradient in volumetric ratios of solvents were as follows: 50–300 mL L^{-1} B over 50 min. The solvent gradient was held at 300 mL L^{-1} B for an additional 15 min and at 65 min the gradient was increased to pure B. It was maintained at pure B for an additional 10 min to clean up the column. Dual wavelengths (270 nm and 325 nm) were used to detect the eluent composition. HPLC analysis at 270 nm was used for quantification of the peak areas of individual phenolic acids. For total phenolic acids measurements sum of peak areas of individual phenolic acid were used for calculations.

Approximately, 500 ± 1 mg of freeze-dried and powdered black cohosh was extracted with solvent of varying polarity (MeOH:H_2O, 100:0, 80:20, 60:40, 50:50, 0:100, v/v) in 11-mL stainless steel cells with PLE using the same operating conditions as described previously. The extracts were transferred to volumetric flasks and volume was adjusted to 25 mL. Five milliliters of the extract were dried under a nitrogen stream at ambient temperature. The dried residue was saponified by stirring the residue at 40 °C for 30 min with 5 ml of NaOH (2 mol L^{-1}) solution containing EDTA (0.1 mol L^{-1}) and ascorbic acid (10 mg mL^{-1}). The pH of the saponified extracts was adjusted to 3 by adding 7 mol L^{-1} HCl. Hydrolyzed phenolic acids were extracted with ethyl acetate (3 × 5 mL). The upper organic layer was removed and evaporated to dryness under nitrogen. The dried residue containing free phenolic acid was redissolved in 2 ml of 750 mL L^{-1} aqueous MeOH and analyzed by HPLC and LC-MS.

Identification of phenolic acids by LC-MS

An Agilent 1100 LC system coupled with a diode array and MSD (SL) detector (Agilent, Palo Alto, CA, USA) was used to identify individual phenolic acids. For LC-MS analysis, the same column, flow rates, and gradients were used as described for HPLC. Mass spectra were acquired in the positive ion mode at both low and high fragmentor voltages (70 V and 250 V). The instrument was set to scan from 100 to 2000 mass units. The temperature of the drying gas was 350 °C at a flow rate of 13 L min^{-1} and a nebulizer pressure of 50 psi. The LC system was directly connected to the mass spectrometer with no stream splitting. Phenolic acids identification was achieved by comparison of the LC-MS data with known standards and data reported in the literature.

RESULTS AND DISCUSSION

The variability of phenolic compounds is a well-established phenomenon, considering that their biosynthetic origin often stems from adaptation to environmental influences. Hence, to avoid uncontrollable environmental variation, all experiments were performed with the same homogenous batch of black cohosh.

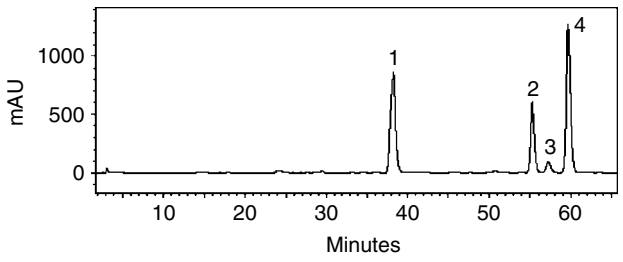

Figure 1. typical HPLC chromatogram with diode array detection showing phenolic acids profile of the saponified black cohosh extract.

Identification of phenolic acids by LC-MS

Figure 1 shows a typical HPLC chromatogram of phenolic acids obtained from saponified black cohosh extract. The structures of the major phenolic compounds were assigned on the basis of a comparison of retention times, UV spectral analysis and spiking with known standard phenolic acids: caffeic acid (compound 1), ferulic acid (compound 2), sinapic acid (compound 3) and isoferulic acid (compound 4). Structures of phenolic acids were confirmed by LC-MS analysis. Compound 1 with an ion at *m/z* 181 $(M + H)^+$ in the positive ion mode and an ion at *m/z* 179 $(M - H)^+$ in the negative ion mode, was confirmed to be caffeic acid. Compounds 2 and 4 showing molecular ions at *m/z* 195 $(M + H)^+$ in the positive ion mode and an ion at *m/z* 193 $(M - H)^+$, were identified as ferulic and isoferulic acids.

Solvent selection

The most common extraction solvent reported in the literature for black cohosh is MeOH.[19,22,23] Kennelly *et al*. had extracted black cohosh with 800 mL L^{-1} MeOH.[24] In the cases where the extract was used for medicinal or ingestion purposes, pure EtOH or a mixture of EtOH and H_2O has typically been used.[18,25] A comparison of the TP extracted from black cohosh was carried out with several different neat solvents such as MeOH, EtOH, deionized H_2O, ACN, acetone, THF, DMSO, as well as a series of MeOH and H_2O mixtures. Since temperature is a variable that affects equilibrium conditions, all measurements were carried out at 40 °C. Use of neat DMSO resulted in the highest TP extraction (16.2 mgGAE g^{-1}), compared with MeOH (6.43 mgGAE g^{-1}) and EtOH (1.9 mgGAE g^{-1}), as shown in Fig. 2. However, use of DMSO is problematic due to its odor and high boiling point. In addition, it is difficult to evaporate DMSO from saponification reaction mixtures and other extracts.

Addition of water is known to cause the plant material to swell thereby allowing the solvent to penetrate more easily in the solid matrix and increase extractability.[26] Figure 3 shows the variation in extraction efficiency of TP with different MeOH:H_2O solvent mixtures. The optimum TP (19.35 mgGAE g^{-1}) was obtained with MeOH:H_2O (60:40, v/v). We also examined this aqueous effect

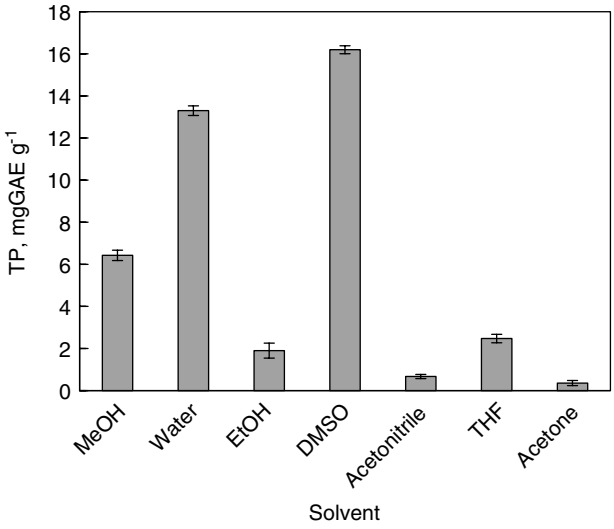

Figure 2. Influence of neat solvents on extractability of TP from the black cohosh matrix.

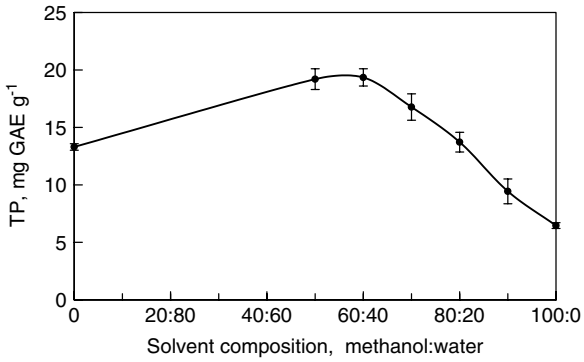

Figure 3. Solvent composition on extractability of TP; influence of various methanol and water mixtures on extraction efficiency.

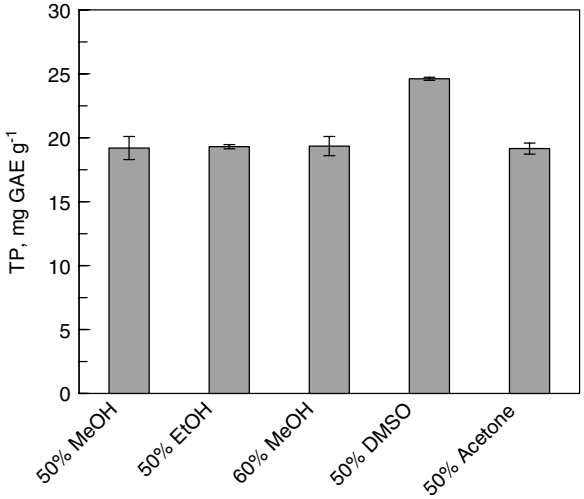

Figure 4. Comparison of added water to various solvents on the extraction efficiency of phenolic compounds.

by employing EtOH:H_2O mixture (50:50, v/v) and obtained a TP value of 19.30 mgGAE g^{-1} compared with 1.9 mgGAE g^{-1} for pure EtOH and

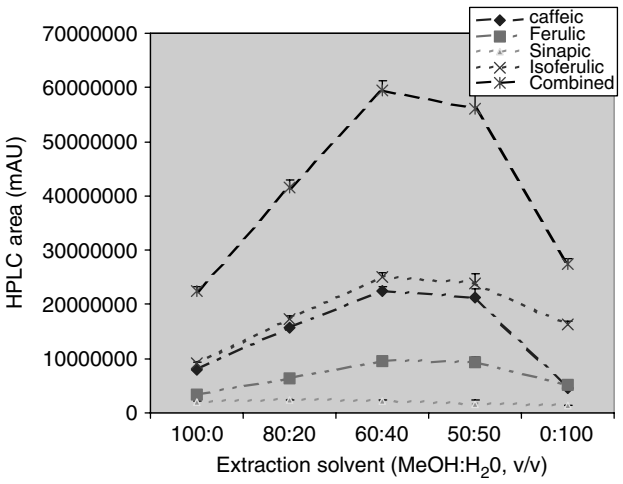

Figure 5. Influence of solvent composition on the extraction efficiency of phenolic acids (HPLC area) with 5 different solvent composition mixtures (MeOH:H_2O, 100:0, 80:20, 60:40, 50:50, 0:100 v/v) as analyzed by HPLC.

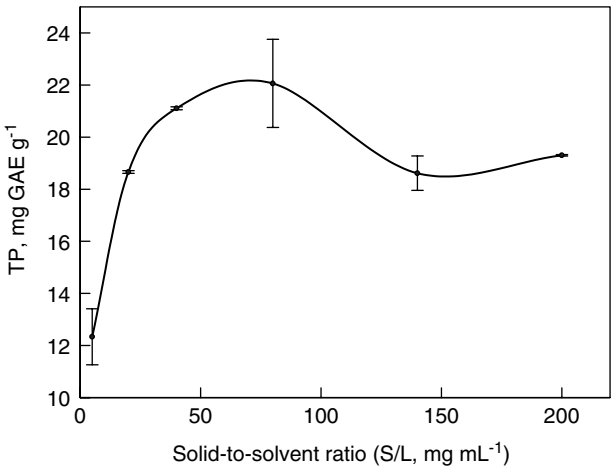

Figure 6. Influence of solid-to-solvent (S/L) ratios on the extraction profile of phenolics from black cohosh.

13.30 mgGAE g^{-1} for neat H_2O. The extraction efficiency of black cohosh with MeOH:H_2O (60:40, % v/v) was found to be similar to that with EtOH:H_2O (50:50, v/v) and acetone:H_2O (50:50, v/v) (Fig. 4). MeOH:H_2O (60:40, v/v) solvent was selected for optimizing additional PLE parameters for extracting TP from black cohosh.

The results obtained for the extraction efficiency of TP by FC assay with varying MeOH:H_2O solvent mixtures were confirmed by HPLC analysis (peak areas). Optimum extraction yields for all phenolic acids were obtained when extraction was carried out with a MeOH:H_2O (60:40, v/v) solvent mixture. Extraction efficiency of other MeOH:H_2O solvent mixtures were calculated by dividing the HPLC peak areas of phenolic acid from different MeOH:H_2O proportions with the HPLC areas of phenolic acids from MeOH:H_2O (60:40, v/v). Comparable yields (94.2%) were obtained with MeOH:H_2O (50:50, v/v) solvent mixture. Only 70% of total phenolics acids were extracted with 80% MeOH. However, pure MeOH or pure H_2O gave extraction efficiencies of total phenolic acids of less than 50%. Similar trends were observed with individual phenolic acids (Fig. 5).

Number of extractions

Using MeOH:H_2O (60:40, v/v) as a solvent, we carried out sequential extractions to find optimal conditions with this variable. Results indicate that nearly 87% of the phenolics ($TP = 17.90 \pm 0.61$ mgGAE g^{-1}) were extracted in the first extraction cycle. An additional 8.8% of the phenolics ($TP = 1.81 \pm 0.05$ mgGAE g^{-1}) were obtained with the second extraction cycle. Approximately 42% phenolics ($TP = 0.86 \pm 0.05$ mgGAE g^{-1}) were obtained in the third cycle. On the basis of these results we chose to use two extraction cycles for the rest of our experiments.

Solid-to-solvent (S/L) ratio

The influence of S/L ratio on the extraction of black cohosh was examined by plotting extraction efficiency in terms of mg of GAE per unit mass of black cohosh against S/L ratio (Fig. 6). Although S/L ratios have not been discussed previously, we calculated from published data that both Li *et al*.[19] and Ganzera *et al*.[23] employed a S/L ratio of 30 mg mL^{-1}. However, no study describing the optimization of S/L ratio has been reported.

The extraction cells available with the PLE equipment (ASE 200) have a fixed volume. We therefore varied the amount of black cohosh from 0.165 to 6.6 g to obtain a range of S/L ratios from 5 to 200 mg mL^{-1}. Using the S/L ratio of 30 as reference,[19,23] we looked at S/L ratios ranging from 5 to 200 (Fig. 6). These experiments were performed with MeOH:H_2O (60:40, v/v) using two extraction cycles.

The TP extracted per gram of black cohosh samples was initially low (e.g. for an S/L of 5, the TP measured was 12.34 mgGAE g^{-1}) and it increased gradually with the increase in S/L ratio until an optimum was achieved. The optimum S/L ratio for extraction was around 80 mg mL^{-1}. It is important to consider and perhaps examine the S/L ratio to exploit maximum extractability while scaling up or down the sample preparation method.

Temperature

The influence of temperature on extraction was investigated since it affects both the equilibrium (solubility) and mass transfer rate (diffusion coefficient).[26] The PLE process allows use of temperatures well above the normal boiling point of the solvent, which is not possible with Soxhlet and other common extraction procedures. Black cohosh (0.660 g) was subjected to extraction with MeOH:H_2O (60:40, v/v) solvent system at six different temperatures: 40, 50, 60, 70, 80 and 100 °C (Fig. 7). All other variables were the same as in the default setting. The maximum TP extraction was achieved around 90 °C. The extraction

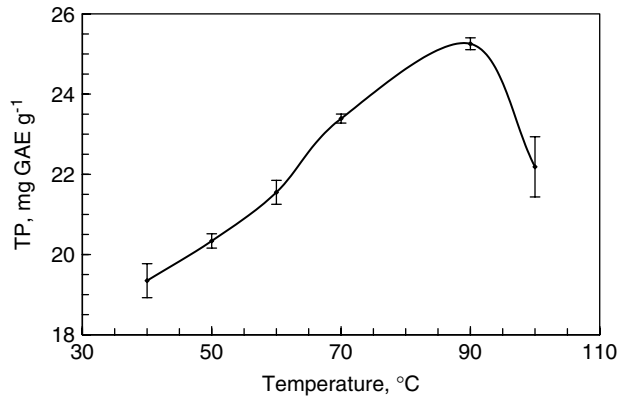

Figure 7. Influence of temperature on the extraction efficiency of phenolic compounds from black cohosh in a pressurized liquid extractor (PLE).

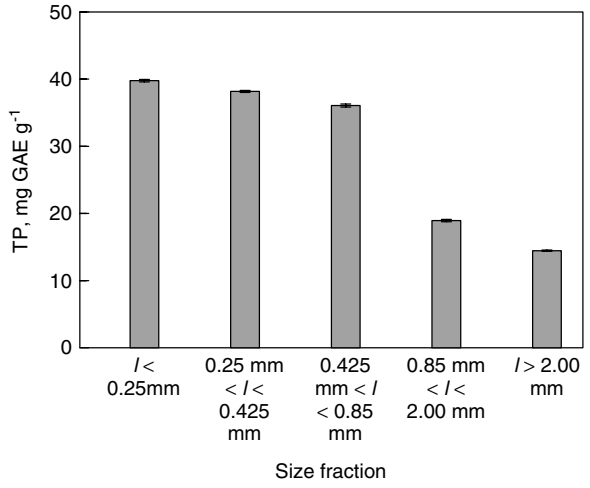

Figure 8. Influence of matrix particle size distribution on the extraction efficiency of phenolic compounds.

efficiency increased by almost 30% as the temperature increased to 90 °C (25.29 ± 0.15 mgGAE g^{-1}) from 40 °C (19.41 ± 0.43 mgGAE g^{-1}). However, at 100 °C, a 20% decline in TP was observed. Therefore, we did not continue the experimentation above 100 °C. In our experiments over 40 °C, we observed the formation of dark brown or black colored precipitates in the extracts. This phenomenon has been observed by other authors.[13] The extent of precipitation or turbidity increased with temperature. It has been reported that the phenolic compounds in grapes, extracted using PLE at high temperature (>100 °C), are quite stable even for most oxidizable phenolics.[27] The highest levels of anthocyanins, phenolics, and antioxidant capacity (as determined by oxygen radical absorbance capacity) have also been reported in grape extract obtained by high-temperature PLE extraction.[28] The most probable cause for a higher TP value at high temperature is the breakage of bonds between various phenolics (analytes) and the plant matrix.[27] On the basis of the data in Fig. 7, we selected 90 °C as the optimum temperature for maximum recovery of phenolics from black cohosh.

Pressure

The high pressure of PLE allows solvents to remain as liquids while working above their atmospheric boiling point. Pressure also aids solvent penetration into sample matrix and move fluids rapidly through the system.[29] Extraction experiments were carried out at three different pressures (500, 1000, and 1500 psi). All other PLE extraction parameters were kept unchanged. Operating pressure had very little or no effect on the overall extraction. Values ($n = 3$) of TP obtained at these three pressures were 17.68 ± 0.29, 18.14 ± 0.68 and 17.82 ± 0.22 mgGAE g^{-1} respectively. We chose the median pressure, 1000 psi, to remain consistent with other literature reports.[13]

Particle size distribution

Figure 8 demonstrates that the recovery of phenolics from the black cohosh plant matrix is strongly influenced by variations in the matrix particle size. There was an almost threefold increase in the extraction efficiency as the particle size (l) decreased from greater than 2.00 mm ($l > 2.00$ mm) to less than 0.25 mm ($l < 0.25$ mm). Thus, the black cohosh plant material should be ground to a smaller particle size, as permitted by the process, in order to achieve maximum extraction efficiency. Although often not discussed in extraction experiments, particle size has an impact on the TP extraction. The surface area per unit mass of plant material increases as the particle size decreases, and this influences solubility. In plant materials, the migration of the analytes through the pores on the surface of the particles also dictates the pace and efficiency of extraction. Therefore, as expected, a smaller particle will have a shorter path for the analyte to travel to reach the surface and hence have a higher extraction efficiency.[26]

CONCLUSIONS

The influence of various parameters on the extraction of phenolic acids from freeze-dried black cohosh powder was studied. Neat solvents were investigated. Addition of water to all solvents was found to increase the TP extraction by causing the plant material to swell, thus allowing the solvent to penetrate the solid particles more easily. A mixture of MeOH with H_2O at a ratio of 60:40 (v/v) was the best choice among other MeOH:H_2O solvent compositions evaluated in this study. The structures of the four major phenolic acids in black cohosh were identified by HPLC and LC-MS analysis as caffeic acid, ferulic acid, sinapic acid and isoferulic acid. The concentration of TP per g of black cohosh in the extract was found to depend on the S/L ratio. The optimal S/L ratio for recovery of phenolic acids from black cohosh with MeOH:H_2O solvent mixture (60:40, v/v) was 80 mg mL^{-1}. Maximum extraction efficiency was achieved at 90 °C. Particle size distribution was observed to play an important

role on the extraction and should be considered when quantifying analytes from plant matrices.

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